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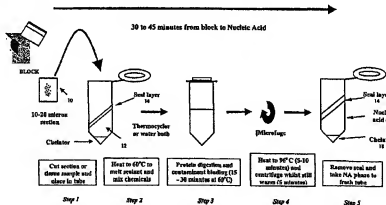
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(54) Title: NUCLEIC ACID EXTRACTION METHOD AND KIT

Archival Block Extraction Process (PCT-Ex) for Paraffin Embedded Tissues
DNA and RNA Extraction for PCR/RT-PCR



(57) Abstract: The present invention relates to a method and a kit for isolating nucleic acids from tissue samples. In particular, but not exclusively, formalin fixed paraffin embedded tissue samples. The isolated nucleic acids will normally be for use in a polymerase chain reaction (PCR). The inventive method comprises the sequential steps of: a) placing in a container the tissue sample, and a wax, a detergent and a tissue digestion enzyme; b) heating the container to a first temperature; c) raising the temperature of the container to a second temperature; d) cooling the container; e) forming a waxy-layer containing impurities and a nucleic acid containing layer; f) removing the waxy-layer, such that the nucleic acids are usable in a polymerase chain reaction. Kits are also provided for performing the same.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Nucleic Acid Extraction Method and Kit

The present invention relates to a method and a kit for isolating nucleic acids from tissue samples. In particular, but not exclusively, formalin fixed paraffin embedded tissue samples. The isolated nucleic acids will normally be for use in a polymerase chain reaction (PCR).

Pathological specimens are routinely taken from patients for use in disease diagnosis and the study of disease marker patterns. Teaching hospitals, medical schools and universities have stores containing millions of such samples, some of which date back over 30 years. These samples represent a major and, at present, an under-used resource for the study of disease progression, the detection of viral, bacterial or parasitic organisms, DNA abnormalities or the detection of genetic diseases.

The specimens are invaluable for the study of many disorders, allowing diagnostic and prognostic indicators to be evaluated. The extraction of nucleic acids from archival samples allows a retrospective genetic and genomic (expression) analysis of the disease state and the correlation of clinical end-points with histological, genetic and genomic (expression) markers.

However the use of archival pathological specimens in genetic association and gene expression studies has been limited. This is due, in the main, to difficulties in obtaining adequate quantities of nucleic acids which are of satisfactory length and integrity for use in analysis. Nucleic acid fragmentation can occur during the fixation process and/or during the extraction of the nucleic acid from the fixed tissue. The ability to extract DNA capable of being amplified by the polymerase chain reaction depends on the duration of fixation of the tissue and the fixative

used. DNA extracted from samples fixed in Bouin's solution is extremely difficult to amplify, possibly due to inhibition of Taq polymerase activity and certain by-products of tissue degradation, e.g. haemoglobin breakdown products and metal ions (Fe^{++}), also affect polymerase activity.

Many pathological specimens, for example archival blocks and pathological slides, are chemically fixed to retain the tissue architecture and especially the conformation of the proteins *in situ*. The use of formalin fixation and paraffin embedding to fix and preserve tissue samples taken from biopsies, resections and smears is almost universal. Whilst the fixatives commonly used effectively preserve the structure of the proteins, the extraction of nucleic acids from the specimens can be difficult.

Techniques for nucleic acid extraction commonly use three separate steps: 1) de-paraffinisation; followed by 2) digestion of the tissue; and 3) purification of the nucleic acids.

Most deparaffinisation methods are based on extraction of the paraffin wax with a solvent, usually xylene. The methods are based on the use of multiple step treatments with xylene followed by treatment with an alcohol such as ethanol and/or another alcohol or a solvent such as acetone. These methods are time consuming and labour intensive and frequently yield highly degraded nucleic acid molecules (RNA in particular) that may not be suitable for analysis e.g. by the PCR and RTPCR (Reverse Transcription PCR). The dissolution of wax in xylene and ethanol is described in Goelz et al., Biochemical and Biophysical Research Communications pages 118-126, Vol 130 No.1, 1985. WO-A-9621042 discloses primers for the PCR amplification of metastatic sequences in fresh or fixed biological samples.

European Patent Application EP-A-0 692 533 discloses a method of treatment of paraffin embedded tissue for gene analysis by extraction of DNA, involving heating a deparaffinized tissue sample obtained from a paraffin-
5 embedded tissue sample at 60°C in an aqueous suspension containing a surfactant having a protein-denaturational action. Alternatively the process further comprises the use of a protease.

10 Since 1985 alternative methods have been developed which include melting the wax in a microwave oven, removal of the paraffin by centrifugation, digestion with Proteinase K and heating to destroy the Proteinase K activity, Bannerjee et al. (1995) Biotechniques 18:768-773. Direct digestion of
15 the tissue has been used by de Lamballerie et al. (1994) J. Clinical Pathology 47: 466-467.

Digestion of the deparaffinised tissue with Proteinase K improves the yield of DNA and prolonged digestion, from 3
20 hours to in excess of 4 days, has been found to improve the yield of high molecular weight DNA using these methods.

European Patent Application EP-A-0 953 635 discloses an improved method for extracting nucleic acids (DNA) from
25 tissue samples and paraffin-embedded tissues.

Cantlay et al. (1994) Thorax 49:1010-1014 and Smith et al. (1997) The Lancet 350:1553-1554 disclose a method of
30 extracting PCR template quality DNA from sections of archival blocks and slide mounted sections (2 to 20 um thickness) using a rapid Proteinase K digestion method.

It is particularly difficult to reproducibly extract RT-PCR template quality mRNA, even in small amounts, from embedded
35 tissue. Using standard solvent based methods the extracted RNA is unlikely to be of the quality required to produce

complimentary DNA (cDNA) copies of greater than 100-150 base pairs when used in reverse transcription assays.

5 This has necessitated the use of other methods, for example
in situ hybridisation to detect discrete mRNA species
directly on slides. The detection of the mRNA being
commonly achieved using radioactively-labelled,
fluorescently-labelled or stainable oligonucleotides or
10 cDNA probes. The utility of this method is limited because
the archival slides can only be reprobbed for different RNA
species a few times, typically two or three times.

15 In order to overcome this restriction a variety of
different fluorophores and quenchers have been developed to
allow the simultaneous detection of several different mRNA
species in one assay.

20 In contrast reverse transcription-PCR (RT-PCR) has fewer
limitations and allows the investigator to detect and
analyse many different mRNA species in single and
multiplexed reactions if the RNA is of fair to good
quality. Near quantitative PCR and RT-PCR are now feasible
and allow the determination of "steady-state" mRNA levels
25 and differential mRNA expression between samples and
between normal and abnormal areas of a single section
following possible micro-dissection of the section.

The present invention seeks to provide a simple method to
extract nucleic acids from tissue samples.

30

According to a first aspect of the present invention there
is provided a method for isolating nucleic acids (DNA and
RNA) from a tissue sample comprising the sequential steps
of:

35 a) placing in a container the tissue sample, a wax, a
detergent and a tissue digestion enzyme;

- b) heating the container to a first temperature;
- c) raising the temperature of the container to a second temperature;
- d) cooling the container;
- 5 e) forming a waxy-layer containing impurities and a nucleic acid containing layer;
- f) removing the waxy-layer, such that the nucleic acids are usable in a polymerase chain reaction.

10

Advantageously the method minimises the number of manipulations in which the container is required to be opened thereby preventing loss of the sample or the aerosol contamination with exogenous nucleic acids or nucleases.

15

A further advantage is that the method also allows removal of proteins, proteases, nucleases and other chemical agents such as heparin, bilirubin, haemoglobin etc which may inhibit the PCR reaction when the waxy-layer is removed.

20

A yet further advantage of the method is that the constant high temperature of the reaction acts to inhibit endogenous tissue nucleases and cause increased disruption of cell membranes increasing the amount and integrity of the nucleic acids which are released from the cells and tissues.

25

The invention thus provides a simple, robust and reproducible nucleic acid extraction (DNA and RNA) method that enables rapid, single and multiple sample extraction.

30

Advantageously the method is useful in the isolation of a wide range of nucleic acids, for example both deoxyribonucleic acids or ribonucleic acids (including poly A RNA) or derivatives thereof.

35

The method thus advantageously provides RNA extraction which is a major advance over prior art methods which are only useful in the isolation of DNA.

- 5 The method allows isolation of nucleic acids capable of being used in the PCR to obtain amplicons with an average size of at least 100 bases, or with an average size of 200 to 500 bases. The isolation of nucleic acids of this integrity therefore has the advantage that the nucleic
10 acids are more likely to be able to act as a PCR template in a PCR reaction and/or RTPCR reaction. Consequently tissue samples which would previously not have been able to provide informative results after assay due to nucleic acid degradation during storage are more likely to provide
15 informative results. The ability to produce RNA of this size is particularly advantageous.

- The tissue sample is preferably selected from liver, uterus, stomach, pancreas, lymphoid tissue, lung, colon,
20 breast, bladder, brain, kidney, bone and blood. The method may also be adapted to use with other types of biological sample, which in the application are referred to generally as tissue samples. The present method thus has the advantage that a wider range of types of tissue samples can
25 be analysed than by prior art methods.

- The tissue sample is fixed, typically using formalin or formaldehyde, and is embedded in a support medium, for example paraffin wax. Advantageously the method allows
30 isolation of nucleic acids from samples where prior art methods have not been able to reliably isolate nucleic acids of sufficient length and integrity to be useable in PCR.

- 35 The tissue digestion enzyme is a protease, for example Proteinase K. Advantageously Proteinase K is catalytically

active at a temperature above the melting point of paraffin wax and can be inactivated at a still higher temperature which can normally be achieved by commonly used heating apparatus.

5

In step a) one or more of water, a buffer, a metal ion chelator and a non-ionic detergent is also placed in the container. Advantageously the method will work with a wide range of reaction conditions which allows a variety of different tissue types to be analysed and allows the method to be sufficiently flexible that it can be optimised for different tissue types and preservation conditions.

The metal ion chelator is selected from Chelex, polyvalent metal ion resin (PMIB), AG50W resins, Bio-Rex 70 resin, Chelex 100 or Sephadex (all chelators are cationic and of biotech grade and the resins comprise paired iminodiacetate ions ($R-CH_2N(CH_2COO^-)$ coupled to a styrene divinylbenzene (or other) support. Advantageously different metal ion chelators may be used in the method thereby increasing the flexibility of the method as regards isolation of different nucleic acid species, ability to digest different tissue types and utility of the method with different tissue preservation conditions.

25

The non-ionic detergent is selected from Tween 20 (polyoxyethylenesorbitan monolaurate), Tween 80 (polyoxyethylenesorbitan mono-oleate), Tween 21 (polyoxyethylenesorbitan monolaurate), Tween 81 (polyoxyethylenesorbitan mono-oleate), Tween 40 (polyoxyethylenesorbitan monopalmitate), Tween 60 (polyoxyethylenesorbitan monostearate), Tween 61 (polyoxyethylenesorbitan monostearate), Tween 85 (polyoxyethylenesorbitan tri-oleate), Tween 65 (polyoxyethylenesorbitan tristearate) and IGEPAL Ca630 ((Octylphenoxy)Polyethoxyethanol formally known as Nonidet

P40 Nonyl Phenol ethoxylate). Advantageously different non-ionic detergents may be used in the method thereby increasing the flexibility of the method as regards isolation of different nucleic acid species, ability to
5 digest different tissue types and utility of the method with tissue preservation conditions.

The first temperature is in the range of 55 to 65°C, preferably 60°C. The first temperature is always a
10 temperature above the melting point of the wax, a temperature at which the tissue digestion enzyme is active. The first temperature thereby allows rapid digestion of the tissue and the extraction of nucleic acids of the desired length.

15 The first temperature is maintained for 15 to 2400 minutes, preferably 15 to 240 minutes and more preferably 30 minutes. Advantageously the first temperature is maintained for 15 to 30 minutes which is a time which allows rapid and
20 effective digestion of the tissue sample resulting in release of nucleic acids which can be used in PCR.

The second temperature is in the range of 90 to 99°C, preferably 96°C. Advantageously the second temperature is
25 a temperature where heat inactivates the tissue digestion enzyme.

The second temperature is maintained for 0 to 20 minutes, preferably for 5 minutes. Advantageously the second
30 temperature is relatively short allowing the extraction of the nucleic acids to be completed rapidly.

The container is cooled, by centrifugation from hot, to 0 to 45°C, preferably room temperature (22°C). Advantageously
35 this cooling step allows the wax to partially solidify into a waxy-layer. Impurities are associated with the waxy-layer

which allows them to be easily removed in later steps.

5 The waxy-layer may be removed by a physical separation means, for example by filtration which may involve using an insert placed in the container or by removal with a sterile probe. Advantageously the waxy-layer can be easily removed by physical separation means such as filtration or manual removal with a sterile probe. This allows relatively unskilled persons to perform the method.

10 Alternatively the waxy-layer may be removed by chemical separation means, for example by affinity binding, preferably by binding to a retaining paper or fibre. Advantageously the waxy-layer may be easily removed by
15 chemical separation means such as affinity binding, preferably to a retaining paper or fibre. This again allows relatively unskilled persons to perform the method.

20 The method may further include a step of purifying the nucleic acids by a physical separation method, for example filtration. Alternatively the nucleic acids are purified by chemical separation, for example affinity binding and/or magnetic separation. Furthermore the nucleic acids may be purified using an enzyme, for example incubation with DNase
25 or RNase.

Advantageously the nucleic acids isolated by the present method are suitable for purification by the commonly used methods of purification, allowing relatively unskilled
30 persons to perform the method.

According to a second aspect of the present invention there is provided a kit for isolating nucleic acids using the method of the present invention, the kit comprising:

- 35 a) a wax and a tissue digestion enzyme, and
b) a wax.

The kit advantageously provides all the non-standard reagents required to conduct the method of the first aspect of the invention. Another advantage is that the kit allows the user to purchase reagents specific for their individual applications, for example specially designed tubes or expensive additional reagents which would not be required by the typical user.

The kit may further comprise a container, for example a single tube or multiple tubes and/or a multiwell plate. Further, the tube plates or other substrate for the kit are normally made from plastics materials, glass, ceramics, silica or may be any other suitable material for the extraction to proceed. Advantageously the kit may include a tube having the wax and tissue digestion enzyme present in the tube to further simplify the method for the user. The inclusion of multiple tubes and/or a multiwell plate allows the screening of several samples at the same time. The wax can advantageously be used to seal the other components in the container which allows easy storage and handling of the kit.

The kit may further comprise a reaction solution, for example a buffer and/or water. Advantageously the method will work with a wide range of reaction conditions which allows a variety of different tissue types to be analysed and allows the method to be sufficiently flexible that it can be optimised for different tissue types and preservation conditions.

The kit may further comprise a metal ion chelator, for example Chelex, polyvalent metal ion resin (PMIB), AG50W resins, Bio-Rex 70 resin, Chelex 100 or Sephadex (all chelators are cationic and of biotech grade and the resins comprise paired iminodiacetate ions $(R-CH_2N(CH_2COO^-))$ coupled to a styrene divinylbenzene (or other) support.

Advantageously different metal ion chelators may be included in the kit thereby increasing the flexibility of the method as regards isolation of different nucleic acid species, ability to digest different tissue types and utility of the method with different tissue preservation conditions.

The kit may further comprise a non-ionic detergent, for example Tween 20 (polyoxyethylenesorbitan monolaurate), Tween 80 (polyoxyethylenesorbitan mono-oleate), Tween 21 (polyoxyethylenesorbitan monolaurate), Tween 81 (polyoxyethylenesorbitan mono-oleate), Tween 40 (polyoxyethylenesorbitan monopalmitate), Tween 60 (polyoxyethylenesorbitan monostearate), Tween 61 (polyoxyethylenesorbitan monostearate), Tween 85 (polyoxyethylenesorbitan tri-oleate), Tween 65 (polyoxyethylenesorbitan tristearate) and IGEPAL Ca630 ((Octylphenoxy)Polyethoxyethanol formally known as Nonidet P40 Nonyl Phenol ethoxylate). Advantageously different non-ionic detergents may be included in the kit thereby increasing the flexibility of the method as regards isolation of different nucleic acid species, ability to digest different tissue types and utility of the method with tissue preservation conditions.

Preferred embodiments of the invention will now be described, by way of example only, with reference to the accompanying drawings, in which;

Figure 1 shows agarose gel electrophoresis of DNA extracted from archival blocks in which:
0.6% agarose gel of DNA extracted from archival blocks. The digestion step using 200µg/ml Proteinase K was carried out for varying lengths of time;
Lane 1 contains a standard molecular weight marker, λphage DNA digested with HindIII;

25 μ sections were digested at 55°C for the following times:

Lanes 2-5, 1 hour; Lanes 6-9, 2 hours; Lanes 10-13, 4 hours; Lanes 14-17, 16 hours;

5 For digested for 16 hours and additional amount of Protease was added after 4 hours of incubation.

Figure 2 shows polyacrylamide gel electrophoresis of BAT26
microsatellite PCR of DNA extracted from archival blocks;
10 8% polyacrylamide gel of genomic DNA specific Bat-26 PCR of
DNA extracted from archival blocks; in which:

Lane 1 contains standard molecular weight marker DNA
from phage λ X174 digested with HaeIII;

15 Lane 2 -ve control 1 (No DNA); Lane 3, +ve control
DNA;

Patient A (1987 block) Lanes 4-7, DNA from normal
colon mucosa section; 8-11, DNA from colon tumour
section;

20 Patient B (1994 block) Lanes 12-15, DNA from normal
colon mucosa section; 16-18, DNA from colon tumour
section 1; 19-21, DNA from tumour section 2;

Patient A can be seen to have microsatellite
instability (double band) and so a deficiency in DNA
mismatch repair in the colon tumour;

25 PCR - Bat 26 microsatellite marker. Intron sequences
on Human chromosome 2;

Primers; Upstream 5'-tgactacttttgacttcagcc-3';
Downstream 5'-aaccattcaacatttttaaccc-3'

Amplicon size: 205bp

30 PCR profile: 50°C, 20s; 72°C, 30s; 94°C, 30s

Figure 3 shows polyacrylamide gel electrophoresis of a
beta-actin specific RT-PCR of RNA extracted from
archival blocks, in which

35 8% polyacrylamide gel of B-Actin specific RT PCR of
RNA extracted from archival blocks;

Lane 1 contains standard molecular weight marker DNA from phage λ X174 digested with HaeIII;
Lane 2 +ve control 1; Lane 3, -ve control (no RNA); RNA extracted from 25 m sections (1997 blocks),
5 Lanes 4 -8, no CHCl_3 treatment; Lanes 9-12, with CHCl_3 treatment; RNA extracted from 25 m sections (1995 blocks), Lanes 13-17, no CHCl_3 treatment; Lanes 18-21, with CHCl_3 treatment;
RT-PCR
10 oligod(T) primer used for reverse transcription of 10ml of each sample; 5l of the RT mixture used in the following PCR reaction:
B actin Primers;
Upstream 5'-ctcaggaggagcaatgatcttg-3'; Downstream 5'-
15 ctgggcatggagtctgtgg-3'
Amplicon size: 200 base pairs
PCR profile: 60°C, 30s; 72°C, 30s; 94°C, 30s

Figure 4 shows agarose gel electrophoresis of the effects
20 of DNase and RNase treatment of the nucleic acid extracted from an archival section;
0.8% agarose gel of nucleic acid extracted from archival sections (20 microns) of Liver, Lung and Colon. The nucleic acid was extracted as per the
25 present invention and treated as follows:
A - No treatment; B - DNase 1 treatment; C - RNase treatment; D - Sequential DNase 1 and RNase treatment; Lanes marked m contain molecular size marker, λ phage DNA digested with HindIII;
30 Note that untreated samples show a diverse staining pattern from greater than 23Kb to less than 2Kb. With DNase 1 treatment the deep banding from the well is no longer present. With RNase treatment the deep banding from the well is still present but lower molecular
35 size species (RNA) are no longer present. Treatment with both enzymes removes the majority of staining

species;

- Figure 5 shows a polyacrylamide gel of multiplexed genomic PCR (three gene loci) on DNA extracted from an archival section;
- 8% PAGE gel of multiplexed genomic PCR reaction on DNA isolated from a control blood sample and different liver sections;
- Specific bands for the human Beta globin (268bp), microsomal epoxide hydrolase (210bp) and CYP2C19 (167bp) genes can be seen in all DNA samples extracted from the archival sections; .
- Marker band sizes are given at the left;
- Figure 6 shows a polyacrylamide gel of duplexed RT-PCR (two distinct species) on RNA extracted from an archival section;
- 8% PAGE gel of duplexed RT-PCR reaction on RNA isolated from control samples and archival sections;
- cDNA synthesis was carried out using random hexamers and the PCR was carried out using specific primer pairs for each RNA species;
- Specific bands for the human actin (162bp) and GAPDH (200bp) RNA species can be seen in all RNA samples extracted from the control archival sections;
- Marker band sizes are given at the left; 8% PAGE gel of duplexed RT-PCR reaction on RNA isolated from control samples and archival sections. cDNA synthesis was carried out using random hexamers and the PCR was carried out using specific primer pairs for each RNA species.
- Specific bands for the human actin (162bp) and GAPDH (200bp) RNA species can be seen in all RNA samples extracted from the control archival sections;
- Marker band sizes are given at the left, and

Figure 7 shows a schematic illustration of a preferred embodiment of the present invention.

Appendix 1 shows a working protocol for the method.

5

The present invention is suitable for use with tissues samples or other biological samples which have been chemically fixed, for example with formalin or formaldehyde and have been embedded in a support medium such as paraffin wax.

10

The sample is contacted with the reaction mixture a tissue digestion enzyme. Additional wax may be introduced at this time. Further wax will be required where the tissue sample has little or no wax associated therewith. The preferred waxes include paraffin wax and others known to the person skilled in the art.

15

Suitable tissue digestion enzymes include proteases which are used to break down tissues and proteins thereby helping to release the nucleic acids. The preferred protease is thermostable at the reaction temperature which is a temperature above the melting point of paraffin but is inactivated at a higher temperature. Ideally the protease degrades a wide range of proteins, it is particularly preferred that the protease has activity against nucleases such that degradation may result in the released DNA having a longer length.

20

25

Preferred proteases include Proteinase K (E.C. 3.4.21.64 from *Tritirachium album*) and other proteases known to the person skilled in the art. A preferred enzyme concentration is 100 to 400 ug/ml, a more preferred enzyme concentration is 150-200 ug/ml.

30

Optionally the composition may additionally comprise at

35

- least one of the following to improve the release of nucleic acids: water, a buffer, a metal ion chelator or a non-ionic detergent. The requirement for the use of the optional ingredients depends upon the tissue type or biological sample being digested.

- Preferably the water will be of molecular biology grade [DNase and RNase free and deionised may also be treated with DEPC (diethyl pyrocarbonate) which attacks amine groups and destroys residual enzyme activity, RNase in particular] and may be treated to inactivate nucleases such as RNases if required. If a buffer is used it may be prepared using molecular biology grade water or other solvent known to the person skilled in the art as appropriate.

- Suitable common biological buffers include organic buffers that maintain the pH in the range 4 to 10, preferably 7 to 9. Preferred buffers include glycine, phosphate, MOPS, TRIS and tricine and others known to the person skilled in the art. The amount of buffer used is dependent upon the pKa and is sufficient to maintain the desired pH of the solution when the tissue is being digested.

- A metal ion chelator may be used to complex metal ions which interfere with the activity of the tissue digestion enzyme. The necessity for a metal ion chelator is dependent upon the type of tissue which is being digested, for example when digesting liver tissue a higher concentration of metal ion chelator is preferred than when digesting lung tissue. Suitable metal ion chelators include Chelex 100, polyvalent metal ion resin (PMIB), AG50W resins, Bio-Rex 70 resin, Chelex 100 or Sephadex (all chelators are cationic and of biotech grade and the resins comprise paired iminodiacetate ions $(R-CH_2N(CH_2COO^-))$ coupled to a styrene divinylbenzene (or other) support and other chelators known

to the person skilled in the art. The concentration of metal ion chelator is preferably in the range 0.5 to 5.0% wt/vol.

- 5 A non-ionic detergent is included in the reaction mixture to assist in the degradation of the tissue sample by the tissue digestion enzyme. Suitable non-ionic detergents include Tween 20TM, NP-40TM and others known to the person skilled in the art. The concentration of the non-ionic
10 detergent is preferably in the range of 0.1 to 2 % vol/vol most preferably in the range 0.4 to 0.6% vol/vol.

- The sample is heated to a first temperature sufficient to melt the paraffin wax and maintained at this temperature
15 for a time which allows the tissue digestion enzyme to degrade the tissue and release the nucleic acids from the tissue. If, for example Proteinase K is used as the tissue digestion enzyme and the tissue is e.g. colon, a preferred first temperature range would be 55 to 65°C. A more
20 preferred first temperature would be 60°C. An incubation time would be 15 to 2400 minutes, more preferably 30 minutes.

- The temperature of the sample is then raised to a second
25 temperature sufficient to inactivate the tissue digestion enzyme and maintained at that temperature until the enzyme is inactivated. If, for example Proteinase K is used as the tissue digestion enzyme and the tissue is e.g. colon, a preferred second temperature range would be 90 to 99°C, a
30 more preferred second temperature would be 96°C. An incubation time would be 0 to 20 minutes, more preferably 5 minutes.

- The sample is then cooled, by centrifugation from hot, to
35 0 to 45°C, preferably room temperature (22°C).

5 The optimum reaction conditions of first and second temperature, pH, ionic strength, presence and concentration of metal ion chelator and detergent may differ with the type of tissue and the type of tissue digestion enzyme used but can be determined by the person skilled in the art without undue effort or the exercise of inventive skill.

10 Removal of the waxy-layer is possible by a variety of physical and chemical separation methods. Removal may be by filtration or affinity binding. The removal may be achieved with the use of a tube insert comprising the filtration means or by the binding of the wax to a retaining paper or fibre inserted or present within the container. Manual removal of the wax and protein fraction with a sterile
15 toothpick or pipette is also contemplated as are other methods known to the person skilled in the art.

20 Purification of the nucleic acids is possible by physical, chemical or enzymatic methods. Purification may be by affinity binding. Alternatively enzymatic digestion of contaminating nucleic acid types may be used. The use of spin columns containing a DNA, RNA or mRNA binding matrix is a preferred method of purifying the desired nucleic acid species. The use of magnetic beads coated with an affinity
25 binding substance is also a preferred means of purification. Enzymatic purification methods include incubation with DNase if RNA is required and incubation with RNase if DNA is required are also preferred as are other methods of purification known to the person skilled
30 in the art.

EXAMPLE 1 Basic Protocol

(A) Method For Preferential Extraction Of Total Nucleic Acid (Eukaryotic and Prokaryotic) From The Section or
35 Tissue Sample

Tube Preparation:

1. Prepare a solution of
 - 2% v/v Tween 20 (SigmaUltra Grade)
 - 20% w/v Chelex 100 Resin (Biotech Grade)
 - 5 - 40mM Tris pH8.0
 - 4mM EDTA pH8.0
 - Make up the volume with 0.1% DEPC treated (nuclease free) dH₂O.
 2. Ensure the solution is homogenous throughout, aliquot
10 50Fl into each reaction tube.
 3. Add one sterile wax bead to each tube.
 4. Incubate the tubes at 65°C until the wax has melted.
 5. Quickly transfer the tubes to a non-refrigerated
15 centrifuge (i.e. benchtop) and spin at full speed for 10 minutes.
 6. Store at 4°C until needed.
- (B) Method For The Extraction of Total Nucleic Acid
(Eukaryotic and Prokaryotic) From The Section or Tissue
20 Sample.

Tube Preparation

- 1.8% Tween 20 (Sigma Ultra Grade)
 - 1.8% v/v Nonidet P40 (Sigma Ultra Grade)
 - 100mM Tris pH8.3
 - 25 200mM KCl
 - 8mM MgCl₂
 - Make up the volume 0.1% DEPC treated (nuclease free)
dH₂O
- Ensure the solution is homogenous throughout, aliquot 50μl
30 into each reaction tube.
- Add one sterile wax bead to each tube.
- Incubate the tubes at 65°C until the wax has melted.
- Quickly transfer the tubes to a non-refrigerated centrifuge
(i.e. benchtop) and spin at full speed for 10 minutes.
- 35 Store at 4°C until needed.

Extraction Protocol - as shown in Fig. 7

1. Place the tissue section 10 into a reaction tube 12 on top of the wax layer 14.
2. Add 150Fl of 267Fg/ml Proteinase K (low grade) to the tube.
3. If possible try to ensure the tissue is submerged.
4. Heat the tube to 60°C in a heating block, water bath or thermal cycler.
5. Once the wax 14 has melted (usually around 2 minutes at 60°C) mix vigorously by vortexing. It is suggested at this point that the tissue is immersed in the buffer.
6. Incubate the sample at 60°C for 30 minutes. (The incubation time may be adjusted, for example, between 15 - 2400 minutes preferably 15-240 minutes).
7. Heat the tube 12 to 99°C in a heating block, water bath or thermocycler.
8. Incubate the sample at 99°C for 10 minutes. (The incubation time may be adjusted, for example, between 5 - 20 minutes).
9. Mix the sample vigorously by vortexing and quickly transfer the tube to a non-refrigerated centrifuge (i.e. benchtop) and spin at full speed for 5 minutes. It is suggested that the transfer is performed quickly, if the tubes are allowed to cool too much the wax will not form a solid layer on top of the buffer. If this happens, it is suggested that the samples are reheated to 99°C for one minute and then re-spun.
10. Carefully excise or pierce the wax layer 14 using a sterile pipette tip.
11. Remove the liquid phase to a clean fresh tube 16, taking care not to transfer any Chelex resin 18.
12. Before analysis, centrifuge the nucleic acid to pellet any residual debris, contaminants or Chelex resin 18.
13. Use 0.5 to 5.0Fl in PCR (1 or 2Fl is usually optimal), or 5 to 10Fl for first strand synthesis (then use 5-

10% of cDNA for PCR).

- NE Vortexing the sample intermittently during the incubations may increase the nucleic acid yield slightly due to the additional manual disruption of the tissue by the Chelex beads, but this is by no means essential -
- 5 PCR/RT-PCR quality nucleic acid is obtainable with only two vortexing steps (as above).

EXAMPLE 2 Separation of nucleic acid by enzymatic method.

10

Remove the wax plug from the tube or plate well.
Divide the sample into two appropriately sized aliquots into fresh tubes or plates.
Treat one of these aliquots with RNase to produce a DNA pool and the other aliquot with DNase to produce an RNA pool as follows.

15

RNAse A treatment

Add RNAse A to the samples to final concentration of 20µg/ml and mix thoroughly by inversion.

20

Incubate the samples at 37°C for 30minutes.

Remove protein by 'salting out'.

DNase I treatment

Add sufficient 10X DNase 1 buffer (to make sample a final 1X), 40 units of RNasin (RNAse inhibitor) and 1-2 units of DNase I.

25

Incubate the samples at 37°C for 30 minutes.

Add 0.1 volume of 25mM EDTA (pH8.0).

Remove protein by 'salting out'.

Salting out procedure

30

Add 0.5 volume of 6M ammonium acetate, mix and leave for 5 minutes on ice.

Centrifuge at 14,000Xg for 10 minutes at 18°C.

Transfer supernatant to a fresh tube.

Add 0.1 volumes of 3M Sodium Acetate (pH5.3) and 3 volumes of 95% ethanol.

35

Place tubes at -40°C for 30 minutes.

Centrifuge at 14,000Xg for 10 minutes at 4°C.

Remove the ethanol.

Wash the pellet with 70% Ethanol.

Centrifuge at 14,000Xg for 1 minute at 4°C.

- 5 Remove the supernatant.

Air dry, inverted, for 10-15 minutes.

Re-suspend the pellet in 1mM EDTA, 10mM Tris/HCl (pH8.0) or sterile water.

- 10 Figure 4 demonstrates that this method of treating the final nucleic acid pool released by the present invention does efficiently lead to separate RNA and DNA pools. The banding produced by staining of an electrophoresis gel of the untreated and enzymatically treated samples illustrates the complete degradation or removal of DNA material by
15 DNase and the removal of RNA species by RNase.

EXAMPLE 3 Protocol for Whole Blood

- 20 1. Aliquot 750Fl of cold, nuclease free TE pH8.0 into a 1.5ml tube.
2. Add 25Fl to 100Fl of whole blood and mix well by vortexing.
3. Centrifuge at full speed for 2 minutes at 4°C.
- 25 4. Remove all but 100Fl of supernatant by aspiration, taking care not to disturb the leukocyte pellet.
5. Add 500Fl of cold, nuclease free TE pH8.0 and mix well by vortexing.
6. Centrifuge at full speed for 2 minutes at 4°C.
- 30 7. Remove all but 100Fl of supernatant by aspiration, taking care not to disturb the leukocyte pellet. By now the leukocyte pellet should be clean and white. [It is suggested that if the pellet is still red, steps 5-7 are repeated until it is white].
8. Resuspend the pellet in 150Fl of 267Fg/ml Proteinase K.
- 35 9. Once the solution is homogenous, transfer to a

reaction tube.

10. Continue with the basic protocol at step 4.

5 In our hands the process yields nucleic acid capable of amplification by PCR from every block section and over 90% of slide sections (liver and colon biopsy samples. PCR quality nucleic acids have been obtained from over 95% of 20 micron sections of breast, bladder, lung and kidney archival samples. In contrast lengthy (>24h) classical
10 xylene-based extraction methods yield good quality DNA from less than 20% of block sections.

Figure 1 shows an agarose gel of the nucleic acid extracted from 20 micron sections taken from archival colon biopsies.
15 It is interesting to note that the yield and quality of the nucleic acid is similar whether the incubation to remove protein is carried out for 1, 2, 4 or 16 hours at 55°C. The smearing pattern in the gel lanes runs from approximately 100bp-23 Kb in length with the highest concentration at
20 around 3-4 Kb.

Methodology 1

One 25Fm section of colon is placed in 200Fg/ml Proteinase K (Prot K) and 5% Chelex (final vol 200F1). Digested at
25 55°C with gentle agitation every hour. After 4 hours 40Fg Prot K is added to the samples and digested for 16 hours. Heat samples to 99°C for 10 mins, spin whilst hot then cool to room temperature and remove the wax disc with a sterile
30 pipette tip. Solution is then transferred to sterile tubes for storage.

Figure 2 shows the results of BAT26 PCR comparison carried out on DNA isolated from archival blocks of polyps removed
35 from the gut of a patient with suspected hereditary non-polyposis coli cancer (HNPCC). This microsatellite marker

- (BAT26) is an indicator of instability which can be associated with defective DNA repair (MLH-1 and MSH-2 gene defects) in HNPCC. DNA was extracted from different areas of the same slide section containing normal mucosal tissue and tumour tissue in parallel. The results clearly show that the tumour from patient A carries a defect in mismatch repair whereas the normal mucosa of patient A and the normal mucosa and tumour from patient B does not.
- Furthermore Figure 5 shows the results of multiplexed genomic PCR reactions where primers specific for human beta-globin, microsomal epoxide hydrolase and CYP2C19 genes were mixed and simultaneously amplified from DNA isolated from an archival sections of liver, colon and lung tissue. This illustrates that DNA isolated using the present invention can be used for the detection and characterisation of multiple gene loco.

Methodology 2

- Two 20Fm sections of colon are placed in each tube. The method is the method listed for Figure 1 with a 4 hour digestion with Prot K at 200Fg/ml.
- Lanes 4-7 Patient A DNA from normal colon mucosa (12 year old specimen)
- Lanes 8-11 Patient A DNA from colon tumour (12 year old specimen)
- Lanes 12-15 Patient B DNA from normal colon mucosa (5 year old specimen)
- Lanes 16-18 Patient B DNA from colon tumour (5 year old specimen-block A)
- Lanes 19-21 Patient B DNA from colon tumour (5 year old specimen-block B)
- Figure 3 demonstrates that the present invention also releases RNA from over 90% of block sections, as determined

by the use of beta actin-specific RT-PCR amplification of single stranded cDNA synthesised using oligo dT_n primers. The nucleic acid extracted from all of the sections shown were able to act as a template for RT-PCR and produced the expected actin amplicon of 200 base pairs in size.

Furthermore Figure 6 shows the results of duplexed RT-PCR reaction on RNA from archival sections. The RNA isolated from archival sections of liver and lung tissue were subjected to cDNA synthesis carried out using random hexamers (N₆ where N is A, G, C or T) followed by simultaneous amplification using oligonucleotide primers specific for human actin and GAPDH RNA species primers. This illustrates that RNA isolated using the present invention can be used for the detection and characterisation of multiple RNA species.

Methodology 3

Two 20Fm sections of normal colonic mucosa are placed in each tube. The method is the method listed for figure one with a 4 hour digestion with Prot K at 200Fg/ml except lanes 9-12 and 18-21 which are additionally washed once with chloroform before placing in sterile tubes for storage.

Lanes 4-12 2 year old specimen (1997 block)

Lanes 13-21 4 year old specimen (1995 block)

Please note that the invention may be used on unfixed tissue samples, such as blood.

APPENDIX 1

NUCLEIC ACID EXTRACTION KIT FOR PARAFFIN EMBEDDED TISSUE

5 Intended Use:

This kit is designed to extract total nucleic acid from formalin-fixed, paraffin. embedded tissue. The kit is optimised for extraction from one section up to 20µm in thickness in less than an hour.

10

Kit Contents:

- 4 bags of 25 reaction tubes containing Extraction Buffer.
- 4 tubes containing Powder B.
- 1 bottle containing 20ml of Buffer A. This buffer is sterile so aseptic technique should be used.
- 1 instruction leaflet.

15

Preparation, Storage and Handling:

20

Before use, 4ml of Buffer A should be added to each glass bottle. The resulting solution, Buffer C, should be stored at -20°C. Each 4ml bottle contains enough of Buffer C for 25 extractions.

Unused glass bottles containing Powder B should be stored at -20°C.

25

EXTRACTION PROTOCOL:

Place the tissue section into a reaction tube on top of the wax layer.

30

Add 150µl of Buffer C to the tube.

Centrifuge the tube briefly at low speed to ensure the tissue section is submerged.

Heat the tube to 60°C in a heating block, water bath or thermal cycler.

35

Once the wax has melted (usually around 2 minutes at 60°C) mix gently by vortexing [It is essential at this point that

the section is immersed in the buffer].

Incubate the sample at 60°C for 30 minutes. [The incubation time may be adjusted between 15 - 240 minutes].

Heat the tube to 96-99°C in a heating block, water bath or
5 thermal cycler and incubate for 5 minutes. [The incubation time may be adjusted between 5 - 20 minutes].

Mix the sample vigorously by vortexing and quickly transfer
the tube to a non-refrigerated centrifuge (i.e. Benchtop)
and spin at full speed for 5 minutes. [Transfer speed is
10 essential, if the tubes are allowed to cool too much the wax will not form a solid layer on top of the buffer. If this happens, re-heat the samples to 96-99°C for one minute and re-spin].

Place the samples on ice for 5 minutes to ensure the wax
15 layer is fully solidified. This will make the next step easier.

Carefully excise or pierce the wax layer using a sterile
pipette tip.

Remove the liquid phase to a clean fresh tube, taking care
20 not to transfer any resin or cellular debris.

Before analysis, centrifuge the nucleic acid to pellet any
residual debris, contaminants or resin.

Use 0.5 to 5.0µl in PCR (1 or 2µl is usually optimal), or
5 to 10µl for first strand synthesis.

25

Notes:

After extraction samples MUST be stored at -20°C. Failure
to do this will result in degradation of nucleic acid. If
RNA Extraction is also required, samples should be stored
30 at -80°C.

For isolation of eukaryotic nucleic acid use the RED kit.
For isolation of pathogenic nucleic acid use the BLACK kit.
Vortexing the sample intermittently during the incubations
may increase the nucleic acid yield slightly due to the
35 additional manual disruption of the tissue by the Chelex
beads, but this is by no means essential - PCR/RT-PCR

quality nucleic acid is obtainable with only two vortexing steps (as above).

- Due to the fragmented nature of the isolated nucleic acid, long PCR products may be difficult to obtain. We have
- 5 successfully amplified fragments in excess of 400 base pairs but this is dependent on the template and on the gene in question.

For RT-PCR we recommend the following:

- Pre-treat the sample with DNase I to digest genomic DNA.
- 10 Heat inactivate the enzyme at 96°C for 5-10 minutes.
Use random hexamers in first strand synthesis.
If possible use intron-spanning primers to enable differentiation between gDNA and cDNA PCR products.
Keep amplicons as small as possible. We have successfully
- 15 amplified fragments in excess of 250 base pairs but this is dependent on the template and on the gene in question.

CLAIMS:

1. A method for isolating nucleic acids from a tissue sample comprising the sequential steps of:
 - 5 a) placing in a container the tissue sample, a detergent, a wax and a tissue digestion enzyme;
 - b) heating the container to a first temperature;
 - c) raising the temperature of the container to a second temperature;
 - 10 d) cooling the container;
 - e) forming a waxy-layer containing impurities and a nucleic acid containing layer;
 - f) removing the waxy-layer,such that the nucleic acids are usable in a polymerase chain reaction.
- 15
2. A method according to claim 1, wherein the nucleic acids are deoxyribonucleic acids or derivatives thereof.
- 20
3. A method according to claim 1, wherein the nucleic acids are ribonucleic acids or derivatives thereof.
4. A method according to any of claims 1 and 3,
- 25 wherein the nucleic acid contains a poly adenine terminal sequence.
5. A method according to any of claims 1 to 4, wherein the average size of the isolated nucleic acids in
- 30 at least 100 bases.
6. A method according to claim 5, wherein the average size of the isolated nucleic acids is 3000-4000bp.
- 35 7. A method according to claim 5, wherein the maximum size of the isolated nucleic acids is 23Kb.

8. A method according to any of claims 1 to 7,
wherein the tissue sample is selected from liver, uterus,
stomach, pancreas, lymphoid tissue, lung, colon, breast,
5 bladder, brain, kidney bone, biological samples and blood.

9. A method according to any of claims 1 to 8,
wherein the tissue sample is fixed.

10 10. A method according to claim 9, wherein the tissue
sample is fixed using formalin or formaldehyde.

11. A method according to any of claims 1 to 10,
wherein the tissue sample is embedded in a support medium.
15

12. A method according to claim 11, wherein the
tissue sample is embedded in paraffin wax.

13. A method according to any of claims 1 to 12,
20 wherein the tissue digestion enzyme is a protease.

14. A method according to claim 13, wherein the
tissue digestion enzyme is Proteinase K.

25 15. A method according to any one of claims 1 to 14,
wherein in step a) one or more of water, a buffer, a metal
ion chelator and a non-ionic detergent is also placed in
the container.

30 16. A method according to claim 15, wherein the metal
ion chelator is selected from Chelex, polyvalent metal ion
resin (PMIB), AG50W resins, Bio-Rex 70 resin, Chelex 100 or
Sephadex (all chelators are cationic and of biotech grade
and the resins comprise paired iminodiacetate ions ($R-$
35 $CH_2N(CH_2COO^-)$ coupled to a styrene divinylbenzene (or other)
support).

17. A method according to claim 15 or claim 16, wherein the non-ionic detergent is selected from Tween 20 (polyoxyethylenesorbitan monolaurate), Tween 80 (polyoxyethylenesorbitan mono-oleate), Tween 21 (polyoxyethylenesorbitan monolaurate), Tween 81 (polyoxyethylenesorbitan mono-oleate), Tween 40 (polyoxyethylenesorbitan monopalmitate), Tween 60 (polyoxyethylenesorbitan monostearate), Tween 61 (polyoxyethylenesorbitan monostearate), Tween 85 (polyoxyethylenesorbitan tri-oleate), Tween 65 (polyoxyethylenesorbitan tristearate) and IGEPAL Ca630 ((Octylphenoxy)Polyethoxyethanol formally known as Nonidet P40 Nonyl Phenol ethoxylate).
18. A method according to any one of claims 1 to 17, wherein the first temperature is in the range of 55 to 65°C.
19. A method according to claim 18, wherein the first temperature is 60°C.
20. A method according to any one of claims 1 to 19, wherein the first temperature is maintained for 15 to 2400 minutes.
21. A method according to claim 20, wherein the first temperature is maintained for 30 minutes.
22. A method according to any one of claims 1 to 21, wherein the second temperature is in the range of 90 to 99°C.
23. A method according to claim 22, wherein the second temperature is 96°C.
24. A method according to any one of claims 1 to 23 wherein the second temperature is maintained for 0 to 20

minutes.

25. A method according to claim 24 wherein the second temperature is maintained for 5 minutes.

5

26. A method according to any one of claims 1 to 25, wherein the container is cooled to 0 to 45°C.

27. A method according to claim 26, wherein the
10 container is cooled to room temperature (22°C).

28. A method according to any one of claims 1 to 27, wherein the waxy-layer is removed by a physical separation means.

15

29. A method according to claim 28, wherein the waxy layer is removed by filtration.

30. A method according to any of claims 28 or 29, wherein the waxy layer is removed using an insert placed in the container, said insert comprising the physical separation means.

20

31. A method according to claim 28, wherein the waxy
25 layer is removed using a sterile probe.

32. A method according to any of claims 1 to 27, wherein the waxy layer is removed by chemical separation means.

30

33. A method according to claim 32, wherein the waxy layer is removed by affinity binding.

34. A method according to any of claims 32 or 33, wherein the affinity binding is to a retaining paper or fibre.

35

35. A method according to any of claims 1 to 34, further include a step (g) of purifying the nucleic acids are purified by physical separation.

5 36. A method according to claim 35, wherein the physical separation method is filtration.

37. A method according to any of claims 1 to 34, wherein the nucleic acids are purified by chemical
10 separation.

38. A method according to claims 37, wherein the chemical separation method is selected from affinity
15 binding or magnetic separation.

39. A method according to any of claims 1 to 34, wherein the nucleic acids are purified using an enzyme.

20 40. A method according to claim 39, wherein the enzymatic separation method is incubation with DNAase.

41. A method according to claim 39, wherein the enzymatic separation method is incubation with RNAase.

25 42. A kit for isolating nucleic acids using the method of any one of claims 1 to 41, the kit comprising:
a) a wax and a tissue digestion enzyme, and
b) a wax.

30 43. A kit for isolating nucleic acids according to claim 42, further comprising a container.

44. A kit according to claim 43, wherein the
35 container comprises:
1) a single tube; or

- 2) multiple tubes; and/or
- 3) a multiwell plate.

45. A kit according to claim 42, further comprising
5 a reaction solution..

46. A kit according to claim 45, wherein the reaction
solution comprises:

- a) a buffer; and/or
- 10 b) water.

47. A kit according to claim 45, wherein the reaction
solution is selected from water, TE or a buffer containing
a detergent, a metal ion chelator and a protease.

15

48. A kit according to claim 42, further comprising
a metal ion chelator.

49. A kit according to claim 48, wherein the metal
20 ion chelator is Chelex, polyvalent metal ion resin (PMIB),
AGSOW resins, Bio-Rex 70 resin, Chelex 100 or Sephadex (all
chelators are cationic and of biotech grade and the resins
comprise paired iminodiacetate ions ($R-CH_2N(CH_2COO^-)$ coupled
to a styrene divinylbenzene (or other) support.

25

50. A kit according to claim 42, further comprising
a non-ionic detergent.

51. A kit according to claim 50, wherein the non-
30 ionic detergent is Tween 20 (polyoxyethylenesorbitan
monolaurate), Tween 80 (polyoxyethylenesorbitan mono-
oleate), Tween 21 (polyoxyethylenesorbitan monolaurate),
Tween 81 (polyoxyethylenesorbitan mono-oleate), Tween 40
(polyoxyethylenesorbitan monopalmitate), Tween 60
35 (polyoxyethylenesorbitan monostearate), Tween 61
(polyoxyethylenesorbitan monostearate), Tween 85

(polyoxyethylenesorbitan tri-oleate), Tween 65
(polyoxyethylenesorbitan tristearate) and IGEPAL Ca630
((Octylphenoxy)Polyethoxyethanol formally known as Nonidet
P40 Nonyl Phenol ethoxylate).

5

52. A method of isolating nucleic acids substantially
as hereinbefore described with reference to, and/or as
illustrated by the accompanying drawings.

10

53. A kit substantially as hereinbefore described
with reference to, and/or as illustrated by the
accompanying drawings.

Figure 1



Figure 2

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21



Figure 3

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21



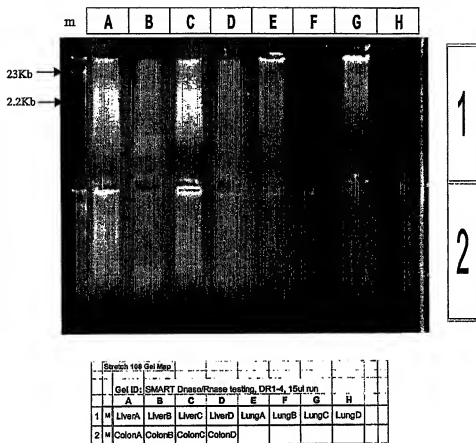


Figure 4

Fig 5



GEL ID: SMART - multiplexing			
Well	marker	Contents	
1	marker		
2	Blood DNA	all primers	
3	Blood DNA	B-globin primers	
4	Blood DNA	mEPHX4 primers	
5	Blood DNA	CYP2C19 primers	
6	Liver Section	all primers	
7	Liver Section	all primers	
8	Colon Section	all primers	
9	Colon Section	all primers	
10	Lung Section	all primers	
11	Lung Section	all primers	
12	negative control	all primers	
13			

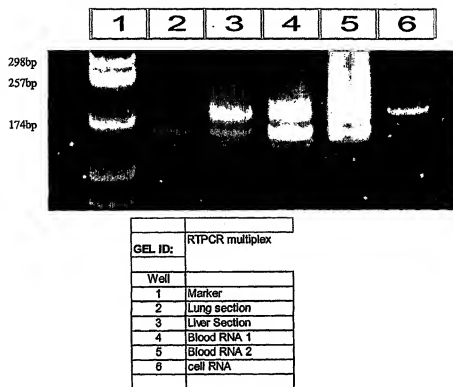


Figure 6

Archival Block Extraction Process (PET-Ex) for Paraffin Embedded Tissue DNA and RNA Extraction for PCR/RT-PCR

